PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Docket No.: **Q84077**
Einar MOEN et al	Conf. No.: **4835**
Appln. No.: **10/511,685**	Group Art Unit: **1657**
Filed: **April 22, 2005**	Examiner: **Singh, SK**

For: **PRODUCT**

DECLARATION UNDER 37 C.F.R. § 1.132

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Gunnar KLEPPE, a Norwegian citizen, hereby declare as follows:

1. I have read and am familiar with the content of the patent application entitled "Product", filed on 17 April 2003 as PCT/GB03/01689, and claiming priority from UK application No. 0209007.4 filed 19 April 2002. This international application subsequently entered the United States national phase as application No. 10/511,685 (hereinafter "The Application"). I have also reviewed the claims (hereinafter referred to as "the amended claims") which I understand are to be filed with this Declaration.

2. I have a professional background as a Biochemist. I was educated at the University of Bergen, Norway to a postdoctoral level. Since 1988 I have been involved in developing large scale biotechnological processes relating to bacterial fermentations, including the development and scale-up of gas-based fermentations which was a particularly challenging project. During the period 1992 to 2006 I was Chief Scientific Officer of the joint Statoil/DuPont company Norferm DA, the company responsible for the development and commercial production of Bioprotein derivatives. As Chief Scientific Officer, I was responsible for product development, product approval and marketing of these Bioprotein derivatives for the feed and

fermentation industries. Today, I serve as a scientific advisor to Bioprotein AS, a university-owned R&D company focusing on the development of novel products from methanotropic bacteria.

The Invention

3. The Application relates *inter alia* to a microorganism growth substrate comprising an autolysed biomass as an organic nitrogen source. The basic biomass is produced from fermentation of *Methylococus capsulatus* (Bath) (strain NCIMB 41526), *Ralstonia* sp. DB3 (strain NCIMB 41527), *Aneurinibacillus* sp. DB4 (strain NCIMB 41528) and *Brevibacillus agri* DB5 (strain NCIMB 41525). Subsequently, this is subjected to an autolytic process as described in The Application whereby to yield an autolysate. The autolysate may be used in "crude" form or, alternatively, may be further processed by methods involving ultrafiltration and evaporation. The pending claims of The Application encompass growth substrates in which the biomass is generated (i) by autolysis of the basic bacterial biomass (hereinafter referred to as "BP Autolysate"); and (ii) by autolysis, ultrafiltration and evaporation of the basic bacterial biomass (hereinafter referred to as "BP Extract").

4. In the growth substrates disclosed in The Application, the autolysed biomass functions as an organic nitrogen source in a complete growth medium which also contains a carbon source, typically glucose. Optionally, the growth substrates can also comprise nitrate and/or mineral salts to aid the growth of microorganisms which require higher levels of salts for optimum growth.

Background

5. The growth substrates which are the subject of the amended claims are versatile for use in culturing a variety of different microorganisms. That this is the case is particularly surprising given the varying nutrient requirements of different bacteria. The growth substrates are especially well suited to the industrial fermentation of lactic acid bacteria such as Lactobacilli. Such bacteria require complex nutrients (vitamins, nucleotides, amino acids and/or peptides) for growth and lactic acid production. Some are also dependent on fatty acids or fatty acid esters for

growth. One standard growth substrate used in culturing Lactobacilli is MRS medium (de Man, Rogosa and Sharpe). This is a complex mixture of proteins from three separate sources (casein, meat and yeast). In contrast to this, it is important to note that the growth substrates of the invention comprise proteins from a single source; this greatly simplifies industrial-scale operations in fermenting Lactobacilli.

6. Not only are the claimed growth substrates "broad-spectrum" substrates for use with different microorganism types, but they can also reduce the cycle time for fermentations and increase the production yield of biomass, expressed proteins and secondary metabolites.

7. The growth substrates which are the subject of the amended claims are commercially available, e.g. as a powdered medium preparation which has a long shelf-life and a high batch-to-batch consistency (see the datasheet provided as Annex 2 for details of the commercial product). The commercial autolysate is free from environmental toxins and genetically modified organisms.

8. The claimed growth substrates are commercially important because they display improved properties for microorganism growth when compared to standard growth substrates used in the art.

Experimental Support

9. The Application includes data from a number of studies that were performed to demonstrate the advantages of the growth substrates of the invention over other growth substrates (culture media) known and used in the art before the priority date. In relation to the autolysed materials, in particular the crude autolysate (i.e. "BP Autolysate"), it is instructive to consider the data in Examples 2 and 6-8 of The Application.

10. Example 2 of The Application describes microorganism growth tests carried out using various growth substrates. The raw data from the tests performed using the "BP Extract" and "BP Autolysate" materials (see Table 1 of The Application) is shown in Annex 1 of this Declaration. These data are summarised in Table A:

	P. aeruginosa	*B. subtilis*	*L. plantarum*	*E. coli*
Characteristics	G(-), aerobe	G(+), aerobe	G(+), anaerobe	G(-), aerobe
Control substrate	CASO	NB	MRS	TSB
BP Extract+glucose	2.70	1.26	1.73	2.48
BP Extract+glucose+salts	2.00	1.19	1.73	2.57
Control	1.00	1.00	1.00	1.00
BP Autolysate+glucose	0.82	1.60	2.71	1.00
BP Autolysate+glucose+salts	1.08	1.40	3.14	1.09
Control	1.00	1.00	1.00	1.00

Table A - growth characteristics of different microorganisms in liquid culture using the growth substrates of the invention. Relative values are shown, i.e. the Optical Density reading (OD_{550}) is proportional to the control value after stationary phase has been reached. A relative value of 2 means that the sample has twice the optical density of the control, which corresponds to approximately twice as many cells, i.e. double the level of growth.

11. The general experimental protocol for the above tests was that volumes of medium (control or growth substrate) in Erlenmeyer flasks were inoculated with the test microorganisms and grown at a given pH and temperature until the culture reached stationary phase. Turbidity (OD_{550}) was regularly measured. The following experimental parameters were used:

	P. aeruginosa	*B. subtilis*	*L. plantarum*	*E. coli*
Details				
Organism I.D.	ATCC 10145	168 *trp2*	ATCC 8014	ATCC 11775
Fermentation vol. (ml)	100	100	200	100
Fermentation temp. (°C)	37	37	37	37
Starting pH	7.3 +/- 0.2	7.0 +/- 0.2	5.7 +/- 0.2	7.3 +/- 0.2
Shaking speed (rpm)	110	200	30	110
Control substrate	CASO	NB (+Trp)	MRS	TSB
BP Extract tests				
Initial OD_{550} (after inoculation)	0.005	0.05	0.1	0.005
Conc. of growth/control substrate (g/L)	0.1	1	4	0.1
BP Autolysate tests				
Initial OD_{550} (after inoculation)	0.1	0.05	0.1	0.005
Conc. of growth/control substrate (g/L)	1	1	4	1

Table B - Experimental parameters used to obtain data in Table A.

12. The control growth substrates used are standard substrates for the bacteria tested. CASO medium (casein-peptone, soymeal-peptone) is an alternative name for TSB medium (Tryptone Soya Broth) as evidenced by data sheet numbers 22092 and 22098 from Sigma-Aldrich (see Annex 3). These data-sheets also indicate that TSB/CASO medium supports the growth of *E. coli* and *P. aeruginosa*. As further evidence that the control growth substrates used for *E. coli* and *P. aeruginosa* are standard substrates, two journal articles are attached as Annexes 4 and 5. The authors of these articles use TSB/CASO media to culture the strains *E. coli* (ATCC 11775) (see page 1, column 1, paragraph 2 of Annex 4) and *P. aeruginosa* (ATCC 10145) (see page 2, column 2, paragraph 2 of Annex 5). MRS medium is the medium recommended for the culturing of *L. plantarum* (ATCC 8014) is evidenced by Annex 6, which is an extract from the ATCC catalogue page for this microorganism (see details under "Growth Conditions"). Nutrient Broth (NB) is a standard medium for the growth of *Bacillus* species (see Annex 7, especially under "DESCRIPTION" on page 223; it should be noted that Nutrient Agar and Nutrient Broth differ only in the presence/absence of agar, which is a gelling agent and not a growth substrate *per se*).

13. Table 1 of The Application is based on data which therefore demonstrates that the "BP Autolysate" and "BP Extract" substrates (with added glucose and, optionally, added mineral salts) are either comparable to the standard media (growth between 0.8 and 1.2 times the growth of the control) or markedly better than the standard media (up to 3.1 times the growth of the control).

14. The significant improvement in the growth of diverse microorganisms on the claimed growth substrates is unexpected and could not have been predicted at the priority date of The Application. Furthermore, the growth substrates of the invention comprise proteins from a single source which is particularly beneficial for large-scale fermentations, especially when compared to the use of standard media such as MRS medium which comprise multiple protein sources.

15. Alongside the improvement in growth of microorganisms on the claimed growth substrates, the data presented in The Application also demonstrates increased viability and productivity of microorganisms grown on such substrates.

16. Example 6 of The Application reports the results of experiments carried out to determine the viability of Lactobacilli in liquid culture. Two strains of *Lactobacillus* were cultured in different media, which were formulated to maintain a constant total nitrogen content. The cell viability for Lactobacilli grown on "BP Autolysate" was in each case around double the viability of the same microorganisms grown on the standard MRS medium (See Tables 7 and 8 on pages 14 and 15 of The Application, in particular the viability (CFU/mL) values for the Media "MRS" and "CA-4"). Furthermore, for both strains of *Lactobacillus* the fermentation time on "BP Autolysate" was reduced compared to the MRS medium, indicating more efficient growth on "BP Autolysate" and a healthier culture (See Table 9 on page 15 of The Application).

17. Examples 7 and 8 report the results of experiments carried out to determine the effect of the growth substrate on enzyme and metabolite production. Figure 2 presented in The Application demonstrates that although the *E. coli* cell density for the "BP Extract" and "BP Autolysate" substrates is lower, the enzyme (β-Gal) activity is comparable to the sample cultured in Yeast Extract, i.e. the relative yield of enzyme per cell is markedly higher for cells grown on the claimed substrate. The data provided in Figure 3 of The Application demonstrates that production of lysine by *C. glutamicum* (NRRL B-11470) cultured in "BP Autolysate" (with added glucose) is significantly higher than production in Bacto Soytone (with added glucose).

18. Similar results to those discussed above have been obtained in separate studies when investigating enzyme expression in *Bacillus* species (keratinase activity) and secondary metabolites in *Streptomyces* species (Natamycin production) using the autolysed materials which are the subject of the amended claims.

19. Thus the claimed growth substrates further function to increase enzyme and metabolite production in many different recombinant microorganisms.

Conclusions

20. The data provided in The Application, as confirmed by the raw data provided herewith, confirms that the growth substrates which are the subject of the amended claims possess improved properties relative to the standard growth substrates used in the art.

21. The claimed growth substrates can support higher cell densities of diverse microorganisms relative to the substrates conventionally known and used in the art. The higher densities are observed for different concentrations of substrate under different conditions (temperature, pH, etc.). As a result, such substrate materials can greatly reduce cycle time and/or increase production yields and thus provide a commercially viable alternative to conventional growth substrates such as Yeast Extracts and Peptones.

22. The growth substrates which are the subject of the amended claims can increase the cell viability of microorganisms relative to standard substrates.

23. The growth substrates which are the subject of the amended claims can increase protein expression and metabolite production in diverse microorganisms.

24. The improved properties of the growth substrates which are discussed herein are unexpected and could not have been predicted at the priority date of The Application. These improvements render the growth substrates commercially valuable.

25. In conclusion, I submit that the growth substrates as claimed in the amended claims possess superior properties relative to the growth substrates used in the art. These advantages are supported by the data provided in The Application and as discussed herein.

26. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Stavanger, Norway on the 22nd day of June 2009

_____

Gunnar KLEPPE

Annex 1

The following figures show the growth of different organisms on various substrates.

The term "Autolysat" in the figure legends corresponds to the "BP Extract" substrate of The Application and the term "Crude Autolysat" corresponds to the "BP Autolysate" substrate of The Application.

The term "NMS" refers to the nitrate mineral salt medium which was used to supplement certain growth substrates. NMS was added at 32.4 ml/L and was prepared as follows:

> 1.0g KNO3 and 0.2g $CaCl_2.2H_2O$ were dissolved completely in water; 1.0g $MgSO_4.7H_2O$, 0.1ml trace elements solution (6.4g $ZnSO_4.7H_2O$, 150mg H_3BO_3, 600mg $CoSO_4.7H_2O$, 130mg $MnCl_2$ and 100mg $NiCl_2.6H_2O$ to 1L in water) and 0.1ml EDTA solution (45g $FeNaEDTA.2H_2O$ to 1L in water) added and then made to 1L with water and autoclaved.

After autoclaving, phosphate buffer (35.6g $Na_2HPO_4.2H_2O$ and 26.0g KH_2PO_4 to 1L in water and autoclaved) was also added to the growth substrates to which NMS was added at 1ml of buffer per 100ml of substrate.

A. *Pseudomonas aeruginosa* (ATCC 10145)

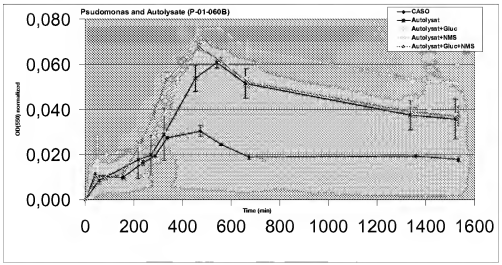

Figure A1 - growth on BP Extract

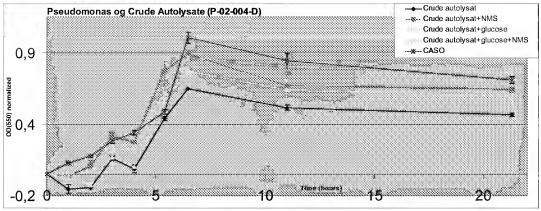

Figure A2 - growth on BP Autolysate

B. *Bacillus subtilis* (168 *trp2*)

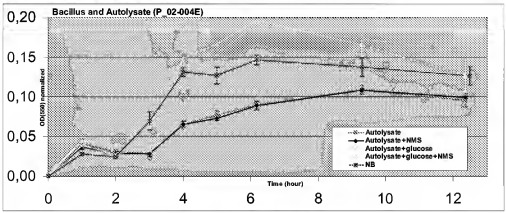

Figure B1 - growth on BP Extract

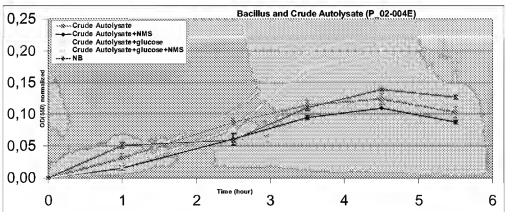

Figure B2 - growth on BP Autolysate

C. *Lactobacillus plantarum* (ATCC 8014)

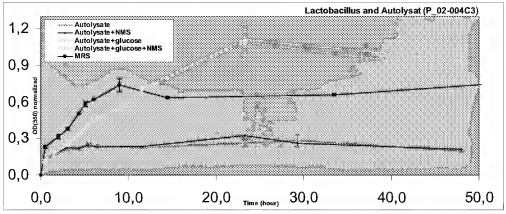

Figure C1 - growth on BP Extract

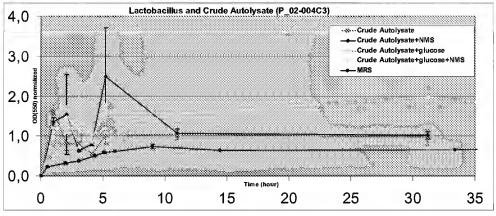

Figure C2 - growth on BP Autolysate

D. *Escherichia coli* (ATCC 11775)

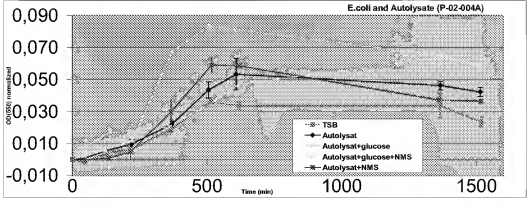

Figure D1 - growth on BP Extract

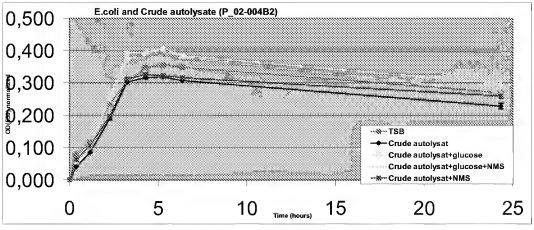

Figure D2 - growth on BP Autolysate

BioProtein®

Your advantage in fermentation production.

Introducing a unique organic stimulant for fermentation production.

BioProtein® Autolysate Product Description

Composition	
Ash	9–11%
Nitrogen	10–11%
Crude lipid	7–9%
Total glucose	4–8%
RNA	3–6%
DNA	1–3%
Total amino acids	50–60%
Free amino acids	10–25%
α-amino acids	3–4%

Amino Acids	
Aspartic acid	45 g/kg
Serine	15 g/kg
Glutamic acid	64 g/kg
Glycine	34 g/kg
Histidine	13 g/kg
Arginine	29 g/kg
Threonine	21 g/kg
Alanine	53 g/kg
Proline	23 g/kg
Tyrosine	22 g/kg
Valine	38 g/kg
Methionine	14 g/kg
Lysine	31 g/kg
Isoleucine	31 g/kg
Leucine	46 g/kg
Phenylalanine	25 g/kg
Cystine	9 g/kg
Tryptophan	12 g/kg

Bioprotein® autolysate advantage at a glance

BioProtein® is a single-cell protein produced from natural gas, oxygen, ammonia, minerals and water. BioProtein® autolysate can be customized for solubility and the amount of free amino acids. It can be produced all year without chemical variation. Performance of BioProtein® is documented through third-party tests, and it has no BSE or GMO relations. It is free from environmental toxins.

Other Data	
Protein digestibility, *in vitro*	85–90%
Protein solubility	30–75%
pH	6.5–7.5
Moisture content	4–8%
Flavor	Neutral
Color	Light brown

Analyzed Vitamins	
Riboflavin (Vitamin B2)	80 mg/kg
Pyridoxine (Vitamin B6)	55 mg/kg
Vitamin K3	10 mg/kg
Vitamin A	250 IE/kg
Biotin	0.5 mg/kg
Niacin	210 mg/kg
Pantothenic acid	40 mg/kg

Other Data	
Protein digestibility, *in vitro*	85–90%
Protein solubility	30–75%
pH	6.5–7.5
Moisture content	4–8%
Flavor	Neutral
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22092 Tryptic Soy Broth (TSB, Tryptone Soya Broth, CASO Broth, Soybean Casein digest Broth, Casein Soya Broth)

The medium will support a luxuriant growth of many fastidious organisms without the addition of serum.
Used for confirmation of *Campylobacter jejuni* by means of the motility test. Recommended by the "Schweizerisches Lebensmittelbuch" 5th ed., chapter 56A, USP XXIII (1995), EP (1999) and the Ph Eur. (1999).

Composition:

Ingredients	Grams/Litre
Casein peptone (pancreatic)	17.0
Soya peptone (papain digest.)	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

Final pH 7.3 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

Directions :
Suspend 30 g of dehydrated media in 1 litre of purified filtered water. Sterilize at 121°C for 15 minutes. Cool to 45-50°C. Mix gently and dispense into sterile Petri dishes or sterile culture tubes.

Principle and Interpretation:
Casein peptone and Soya peptone provide nitrogen, vitamins and minerals. The natural sugars from Soya peptone and Glucose promote organism growth. Sodium chloride is for the osmotic balance, while Dipotassium hydrogen phosphate is a buffering agent.
Tryptone Soya Broth is often for the tube dilution method of antibiotic susceptibility testing. The addition of a small amount of agar (approx. 0.05-0.2% Fluka 05040, add before sterilisation) renders the broth suitable for the cultivation of obligatory anaerobes, such as Clostridium species. The superior growth-promoting properties of Tryptic Soy Broth make it especially useful for the isolation of organisms from blood or other body fluids. Anticoagulants such as sodium polyanetholesulfonate (Fluka 81305) or sodium citrate (Fluka 71635) may be added to the broth prior to sterilisation. 5 to 10 ml of blood may be added to 50 ml of medium.

Cultural characteristics after 18-48 hours at 35°C (if necessary 76 hours).

Organisms (ATCC)	Growth	max. incubation time in days
Escherichia coli (8739)	+++	3
Staphylococcus aureus (6538-P)	+++	3
Streptococcus pneumoniae (6301)	+++	3
Bacillus subtilis (6633)	+++	3
Pseudomonas aeruginosa (9027)	+++	3
Candida albicans (2091 or 10231)	+++	5
Aspergillus niger (6301)	+++	5

References:
1. J.L. Smith, B.J. Dell, Capability of selective media to detect heat –injured Shigella flexneri, J. Food Protect. 53, 141 (1990)
2. R.G. Garison, Studies of the respiratory activity of Histoplasma Capsulatum, J. of infect.. Dis. 108: 120-124 (1961)
3. N.B. Mc Cullough, Laboratory tests in the diagnosis of brucellosis. Amer. J. of puplic health 39: 866-869 (1949)
4. Jean. F. Mac Faddin, Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. Vol. 1. Baltimore, MD.: Williams & Wilkins. (1985)

Fluka
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22098 CASO Broth (Soybean Casein digest Broth, Casein-peptone Soymeal-peptone Broth)

The medium will support a luxuriant growth of many fastidious organisms without the addition of serum.
Used for confirmation of Campylobacter jejuni by means of the motility test. Recommended by the "Schweizerisches Lebensmittelbuch" 5th ed., chapter 56A, USP XXIII (1995), EP (1999) and the Ph Eur. (1999).

Composition:

Ingredients	Grams/Litre
Casein peptone	17.0
Soy peptone	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

Final pH 7.3 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

Directions :
Suspend 30 g of dehydrated media in 1 litre of purified filtered water. Sterilize at 121°C for 15 minutes. Cool to 45-50°C. Mix gently and dispense into sterile Petri dishes or sterile culture tubes.

Principle and Interpretation:
Casein peptone and Soya peptone provide nitrogen, vitamins and minerals. The natural sugars from Soya peptone and Glucose promote organism growth. Sodium chloride is for the osmotic balance, while dipotassium hydrogen phosphate is a buffering agent.
CASO Broth is often for the tube dilution method of antibiotic susceptibility testing. The addition of a small amount of agar (approx. 0.05-0.2% Fluka 05040, add before sterilisation) renders the broth suitable for the cultivation of obligatory anaerobes, such as Clostridium species. The superior growth-promoting properties of CASO Broth make it especially useful for the isolation of organisms from blood or other body fluids. Anticoagulants such as sodium polyanetholesulfonate (Fluka 81305) or sodium citrate (Fluka 71635) may be added to the broth prior to sterilisation. 5 to 10 ml of blood may be added to 50 ml of medium.

Cultural characteristics after 18-48 hours at 35°C (if necessary 76 hours).

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Candida albicans (2091 or 10231)	+++	5
Aspergillus niger (6301)	+++	5

References:
1. J.L. Smith, B.J. Dell, Capability of selective media to detect heat –injured Shigella flexneri, J. Food Protect. 53, 141 (1990)
2. R.G. Garison, Studies of the respiratory activity of Histoplasma Capsulatum, J. of infect.. Dis. 108: 120-124 (1961)
3. N.B. Mc Culloug, Laboratory tests in the diagnosis of brucellosis. Amer. J. of puplic health 39: 866-869 (1949)
4. Jean. F. Mac Faddin, Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. Vol. 1. Baltimore, MD.: Williams & Wilkins. (1985)

Annex 4

fold increase in sensitivity of the Stamm test can he of great importance, however, in the accurate analysis of samples with low peroxide values. The modified Stamm test is slightly more troublesome to carry out than the others, because the reaction mixture must be heated and because the temperature of the solution must be accurately known when the absorbance is determined.

D. L. Hamm[5]
and
E. G. Hammond
Department of Dairy and Food Industry
Iowa State University, Ames

[5] Research and Development Division, National Dairy Products Corporation, Glenview, Illinois.

References

(1) American Oil Chemists' Society. 1964. Official and Tentative Methods. Cd 8-53. American Oil Chemists' Society, Chicago, Illinois.

(2) Driver, M. G., Koch, R. B. and Salwin, H. 1963. Modified Procedure for the Determination of Peroxide Value of Fats by the Ferric Thiocyanate Method. J. Am. Oil Chemists' Soc., 40: 504.

(3) Hamm, D. L., Hammond, E. G., Parvanah, V., and Snyder, H. E. 1965. The Determination of Peroxides by the Stamm Method. J. Am. Oil Chemists' Soc., 42: 920.

(4) Stine, C. M., Harland, H. A., Coulter, S. T., and Jenness, R. 1954. A Modified Peroxide Test for Detection of Lipid Oxidation in Dairy Products. J. Dairy Sci., 37: 202.

Reductive Dechlorination of DDT by *Escherichia coli*[1]

Chlorinated hydrocarbon pesticides are fairly resistant to microbial degradation. The literature on the microbial degradation of chlorinated hydrocarbon pesticides, other than DDT, is limited. Several investigators (1,2,4,6-8) have reported the reductive dechlorination of DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane] to DDD [1,1-dichloro-2,2-bis(p-chlorophenyl)-ethane] by microorganisms. Mendel and Walton (6) suggest that the normal flora of the gastrointestinal tract is the main source of the p,p'-DDD found in animals fed p,p'-DDT, rather than the liver, as suggested by other workers (3).

This study was made to obtain more information on the conversion of DDT to DDD by *Escherichia coli*.

Experimental Procedure

Escherichia coli ATCC 11775 was grown in trypticase soy broth (TSB) for 24 hr at 37 C, and 1 ml was inoculated into each flask of sterile test medium used.

The reductive dechlorination of 77.2% p,p'-DDT, and 99.3% p,p'-DDT was studied in TSB. nutrient broth, brain heart infusion broth, Difco skimmilk, and Matrix. Trypticase soy broth and Difco skimmilk were used to study the reductive dechlorination of 99.3, 97.6, 77.2 p,p'-DDT, Technical DDT, and 70% p,p'-DDD.

Each of the insecticides studied was made up in a stock solution containing 100 mg/100 ml hexane, and 0.1 ml of this solution was added per 100 ml of sterile test medium.

The flasks were incubated at 37 C and analyzed at zero, two, and seven days. Controls containing only the medium and the various DDT's were run and analyzed along with the test flasks.

The sample cleanup procedure of Langlois et al. (5) was used to prepare the samples for injection into the gas chromatograph. The analytical instrument was a Perkin-Elmer Model 811 with an electron capture detector containing a 130-me tritium ionizatiou source. The recorder was a 1-mv Sargent Model SR with a disc integrator unit. The analytical column was a 3.2-mm od by 1.5m Pyrex glass, packed with 5% Dow 11 Silicone on 60/80 mesh Gas Chrom Q. The operating conditions were: 16 DC v across the detector, nitrogen flow of 60 ml/min, and temperatures of 200, 190, and 180 C for the injector, column, and detector, respectively.

The compounds obtained were identified by comparing the retention times with those of known standards injected into the gas chromatograph.

Results and Discussion

Neither the amount nor the structure of the DDT in the controls showed any detectable change after seven days at 37 C.

E. coli ATCC 11775 did not cause a change in the structure of 70% p,p'-DDD when grown either in TSB or in skimmilk. These results are similar to those obtained by other investigators using broth (1, 2, 4, 6).

Figure 1 compares chromatograms obtained after zero and seven days' growth of *E. coli* in TSB containing 99.3% p,p'-DDT. The chromatograms are typical of those obtained from the different broths containing the various DDT's. The results obtained agree with those of Mendel and Walton (6). The p,p' peak of all DDT's studied underwent reductive dechlorination by *E. coli* when grown in the various broths. In general, the p,p' peak was over 50% dechlorinated after two days and over 90% dechlorinated after seven days. Neither the per cent

[1] The investigation reported in this article (no. 67-5-6) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with approval of the Director.

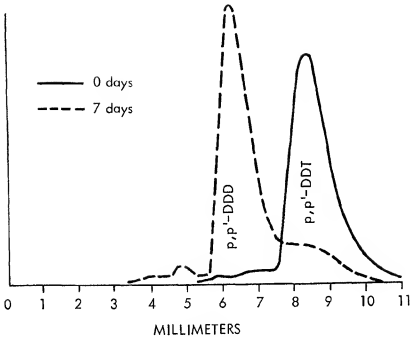

FIG. 1. Chromatograms obtained after growth of *E. coli* in trypticase soy broth containing 99.3% p,p'-DDT.

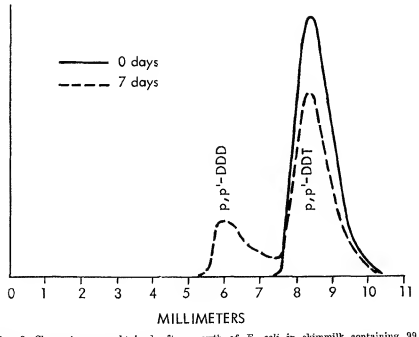

FIG. 2. Chromatograms obtained after growth of *E. coli* in skimmilk containing 99.3% p,p'-DDT.

of the p,p′ isomer nor the type of broth appeared to affect the rate of reductive dechlorination by *E. coli*.

Figure 2 compares chromatograms obtained after zero and seven days' growth of *E. coli* in skimmilk containing 99.3% p,p′-DDT. The chromatograms are typical of those obtained from skimmilk containing the various DDT's. Unlike results obtained with broth, there is little change in the structure of the various DDT's in skimmilk. The p,p′ peak of all the DDT's was not changed after two days and only the 99.3% p,p′-DDT showed a detectable change in the p,p′ peak after seven days. The skimmilk appeared to inhibit the reductive dechlorination mechanism of *E. coli* when used as a growth medium.

Research is now in progress to determine the mechanism of this inhibition in skimmilk.

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Identification of Yellow Material Remaining on Disks After Filtration of Milk [1]

Cole et al. (2, 3) were the first to report that the yellow material appearing on milk sediment-test filter disks was associated with the mastitis condition. Choi and Forster (1) reported a significant relationship among degree of yellow color on milk sediment test filter disks and estalase values, CMT scores, direct microscopic leucocyte count, and A-esterase classes of the same milk samples. Milk carotene content has been shown to increase threefold after interrupted milking (6). Moreover, the greatest fraction of the carotenoids in milk is known to be β-carotene (4). These observations suggested that the yellow color observed by others (1-3) from filtered milk was possibly β-carotene. The purpose of the present study was to identify the yellow material from milk found on disks after filtration.

Methods. Holstein cows from the North Carolina State Experiment Station Herd were used, since milk from this breed normally contains the least amount of carotene (4).

Milk from quarters of five cows comprised the sample analyzed. Quarters were selected on the basis of varying degrees of yellow color both in the whole fresh milk and in the material on Lintine filter disks[2] after filtration, to represent a range in color intensity. Direct microscopic counts showed that these samples ranged from 800,000 to 7,000,000 leucocytes per ml.

The yellow color from the pooled milk sample was extracted by two different methods. In the first method, 15 ml of whole milk at 40 C was added to 50 ml of reagent-grade acetone contained in a 100-ml centrifuge tube. This was mixed for 5 min. To this mixture was added 15 ml of reagent-grade petroleum ether (b.p. 65-100 C) and mixed for an additional 5 min. Samples were then centrifuged at 1,465 *g* for 15 min at 0 C. After centrifugation, the ether layer was drawn off and used for spectrophotometric determination. With this method of extraction, 94% of the yellow material was contained in the first extraction. One additional extraction accounted for another 4%. In the second method, 4 ml of the ether extract (from Method 1) were added to 2 ml of distilled water and 2 ml of 10% alcoholic KOH (95% ethanol). This mixture

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[2] Johnson & Johnson, Chicago, Illinois.

Annex 5

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OPTOMETRY AND VISION SCIENCE

ORIGINAL ARTICLES

Adhesion of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to Silicone–Hydrogel Contact Lenses

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Centro de Engenharia Biológica, Universidade do Minho, Portugal (MH, CS, RO, JA) and Centro de Física, Universidade do Minho, Portugal (ML, ECDRO)

ABSTRACT: *Purpose.* The purpose of this study is to compare the adhesion capabilities of the most important etiologic agents of microbial ocular infection to the recently available silicone–hydrogel lenses with those to a conventional hydrogel lens. *Methods. In vitro* static adhesion assays of *Pseudomonas aeruginosa* 10,145, *Staphylococcus epidermidis* 9142 (biofilm-positive), and 12,228 (biofilm-negative) to two extended-wear silicone–hydrogel lenses (balafilcon A and lotrafilcon A), a daily wear silicone–hydrogel lens (galyfilcon A) and a conventional hydrogel (etafilcon A) were performed. To interpret the adhesion results, lens surface relative hydrophobicity was assessed by water contact angle measurements. *Results. P. aeruginosa* and *S. epidermidis* 9142 exhibited greater adhesion capabilities to the extended wear silicone–hydrogel lenses than to the daily wear silicone– and conventional hydrogel lenses ($p < 0.05$). No statistical differences were found between the adhesion extent of these strains to galyfilcon A and etafilcon A. The biofilm negative strain of *S. epidermidis* adhered in larger extents to the silicone–hydrogel lenses than to the conventional hydrogel ($p < 0.05$), but in much lower amounts than the biofilm-positive strain. The water contact angle measurements revealed that the extended wear silicone–hydrogel lenses are hydrophobic, whereas the daily wear silicone– and conventional hydrogel lenses are hydrophilic. *Conclusions.* As a result of their hydrophobicity, the extended wear silicone–hydrogel lenses (lotrafilcon A and balafilcon A) may carry higher risk of microbial contamination than both the hydrophilic daily wear silicone–hydrogel lens, galyfilcon A and the conventional hydrogel lens, etafilcon A. (Optom Vis Sci 2005;82:446–450)

Key Words: silicone–hydrogel contact lenses, bacterial adhesion, hydrophobicity, *P. aeruginosa*, *S. epidermidis*

Conventional soft lenses based on polyhydroxyethyl methacrylate (p-HEMA) are still the most popular type of contact lenses. These lens materials are copolymers of HEMA and other hydrophilic monomers such as N-vinyl pyrrolidine (NVP) and methacrylates that possess a wide range of water content. The water content is usually above 38 wt%, which contributes to the softness and comfort of these lenses. However, the oxygen permeability of these lenses is limited by the water phase restricting their wearing schedule. The introduction of silicone-containing hydrogel contact lenses having the same comfort and significant higher oxygen permeabilities than conventional hydrogel has resulted in a new generation of soft contact lenses. The high oxygen permeability on account of the Siloxane component makes it possible to wear these lenses on a continuous basis for up to 30 days.[1] Recently, the U.S. Food and Drug Administration approved a new silicone–hydrogel lens for daily wear (galyfilcon A) that combines the high oxygen transmissibility nature of a silicone–hydrogel with the great wettability and flexibility of a conventional hydrogel.[2]

One of the main problems associated with contact lenses is microbial contamination of the lens surface. Although the extended wear contact lens reduces the frequency of handling and thus the risk of contamination, no protection from infection by regular cleaning and disinfection is provided. Although the estimated risk of the incidence of silicone–hydrogel lens-associated keratitis is one in 15,800 patients years,[3] which is approximately 30 times lower than for conventional hydrogels,[4] this fact should not be ignored.[5]

Adhesion of bacteria, notably *Pseudomonas aeruginosa* and *Staphylococci* strains, to contact lenses is considered a primary risk factor of serious corneal problems.[6,7] The contact lenses provide a suitable substratum for bacterial adherence and biofilm formation,[8] supplying an inoculum of organisms in prolonged contact with the cornea. Additionally, the corneal interaction with the contact lens can overwhelm the protective mechanisms of the cornea, increasing the ability of microbial cells to adhere to the cornea and progress to microbial keratitis.[9] The risks associated with silicone–hydrogel lenses, regarding microbial contamination, have not been fully evaluated. In this work, the relative adhesion capability of the most important etiologic agents of microbial ocular infection (*P. aeruginosa* and *S. epidermidis*) to the recently available silicone–hydrogel lenses (lotrafilcon A, balafilcon A, and galyfilcon A) versus a standard hydrogel lens (etafilcon A) was studied.

MATERIALS AND METHODS

Contact Lenses

The silicone–hydrogel lenses used in this study lotrafilcon A (Focus Night & Day; CIBA), balafilcon A (PureVision; Bausch & Lomb), and galyfilcon A (Acuvue Advance, with HYDRACLEAR; Johnson & Johnson) were kindly provided by the manufacturers as well as the HEMA lens etafilcon A (Acuvue; Johnson & Johnson). The contact lenses properties are summarized in Table 1.

Artificial Tears

Artificial tears were prepared with 1.4 g of polyvinyl alcohol (Sigma-Aldrich) and 0.6 g of povidone (Sigma-Aldrich) in 100 mL of a saline solution (0.9% NaCl). The pH of this solution was adjusted to 7.5 with NaOH and sterilized by vacuum filtration through a 0.2-μm filter. Artificial tears were made with the purpose of reproducing physicochemical properties of natural tears, namely pH, ionic strength, and viscosity.

Viability tests based on CFU determinations of bacterial suspensions incubated in artificial tear and a control saline solution (0.9%) demonstrated that artificial tears do not affect the viability of the assayed bacterial strains (data not shown).

Bacterial Strains and Growth Conditions

The strains used in this study were a biofilm-negative *S. epidermidis* ATCC 12,228,[10,11] a biofilm-positive *S. epidermidis,* 9142 and *P. aeruginosa* ATCC 10,145. *S. epidermidis* 9142 is a known producer of the major surface polysaccharide promoting coagulase-negative Staphylococci adherence and biofilm formation, referred to as either polysaccharide intercellular adhesin (PIA) or by its chemical composition, poly-N-acetyl glucosamine (PNAG). This strain was provided by Dr. Gerald B. Pier, Harvard Medical School, Boston. Strains of *S. epidermidis* ATCC 12,228 and *P. aeruginosa* ATCC 10,145 were obtained from the American Type Culture Collection. All strains were stored at -70°C on 25% glycerol.

Staphylococci strains and *P. aeruginosa* were incubated in 10 mL of TSB tryptic soy broth (TSB) during 24 hours at 37°C. After this period, 100 μL of the culture suspension were transferred to 50 mL of fresh TSB and incubated for 18 hours at 37°C to obtain a midexponential growth culture. Cells were harvested by centrifugation (15 minutes, 5000 g) and washed two times with artificial tears.

Adhesion Assays

The method used to assess bacterial adhesion to contact lenses was the static adhesion assay. This method consisted in immersing each contact lens, with the convex side up, in 1 mL of a cell suspension (5×10^8 CFU/mL) prepared in artificial tears and placed in a well of a 24-well tissue culture plate (Sarstedt). The tissue culture plate was incubated for 2 hours at 37°C and after this period, each contact lens was carefully removed from the well with a tiny forceps and washed three times by immersing the lens in clean artificial tears for 15 seconds. This washing step was carefully performed to remove only the cells that were suspended in the liquid interface formed along the surface and to minimize cell detachment from the surface.

After the adhesion assay, two opposite edges of each contact lens were cut to flatten the surface to be mounted on a microscope slide with the correspondent convex side up. Cell enumeration was performed using a phase contrast microscope coupled to a 3-CCD video camera that acquires images at a magnification of 1622 × and 20 images were randomly taken from each contact lens. Cells were enumerated using an image analysis system (SigmaScan Pro5 SPSS). The adhesion experiments were done in triplicate and repeated twice.

Contact Angle Measurements

Relative lens surface hydrophobicity was determined by measurements of water contact angles. Before the measurements, contact lenses were immersed in artificial tears for 30 minutes. The excess of liquid was then removed by gently tapping the side of the lens on a filter paper. Contact lenses were then cut into quarters

TABLE 1.
Summary of contact lens properties

Category	Name, material	Manufacturer	FDA group	Water content (wt.%)	Oxygen permeability (Dk barrera)
Silicon-based	Focus Night & Day, lotrafilcon A	CIBA	I	24	140
	PureVision, balafilcon	Bausch & Lomb	III	36	91
	Acuvue Advance,galyfilcon A	Johnson & Johnson	I	47	60
p-HEMA-based	Acuvue, etafilcon A	Johnson & Johnson	IV	58	28

[a] $\times 10^{-11}$ cm mL O_2/s mL mm Hg.

and each quarter mounted on a microscope slide. Measurements of advancing type water contact angles were carried out on the convex side of a contact lens quarter using the apparatus OCA 20 (Dataphysics). The measurements were performed immediately after cutting the contact lens to avoid lens dehydration and at 25°C. These measurements were repeated eight times per contact lens material.

Statistics

The data obtained was analyzed using a statistical program, SPSS (Statistical Package for the Social Sciences). One-way analysis of variance with Tukey test was used to compare the number of adhered cells for each contact lens type and for each strain. All tests were performed with a confidence level of 95%.

RESULTS

The number of cells of *P. aeruginosa* and *S. epidermidis* (ATCC 12,228 and 9142) attached to the three silicone–hydrogel contact lenses and to the conventional hydrogel lens are present in Figure 1. *S. epidermidis* 12,228 adhered in larger extent to the silicone–hydrogel contact lenses than to the conventional hydrogel ($p < 0.05$). Conversely, no statistical differences were found between the adhesion of *S. epidermidis* 9142 and *P. aeruginosa* to galyfilcon A and etafilcon A, although the adhesion of these two strains to lotrafilcon A and balafilcon A occurred in greater amounts ($p < 0.05$).

Comparing the adhesion behaviors of the three strains, it was found that the number of adhered cells of *P. aeruginosa* to etafilcon A was significantly higher than that of *S. epidermidis* 12,228 ($p = 0.002$) and *S. epidermidis* 9142 ($p = 0.005$). There were no statistical differences in the adhesion extents of all strains to galyfilcon A. Considering the adhesion to lotrafilcon A and balafilcon A, the number of adhered *S. epidermidis* 12,228 was significantly lower than the number of adhered *S. epidermidis* 9142 ($p = 0.01$ and $p = 0.045$, respectively, to each contact lens) and the number of adhered *P. aeruginosa* 10,145 ($p = 0.003$ and $p = 0.004$, respectively, to each contact lens).

Figure 2 presents the values of the water contact angles measured on the lens surfaces. According to van Oss and Giese,[12] material surfaces can be considered hydrophobic if the water contact angle is higher than 50°. Considering this notation balafilcon A and lotrafilcon A are hydrophobic, whereas galyfilcon A and etafilcon A are hydrophilic.

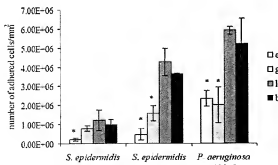

FIGURE 1.

Number of adhered cells of *Pseudomonas aeruginosa* 10,145, *Staphylococcus epidermidis* 9142, and *S. epidermidis* 12,228 per mm² to each type of contact lens. The asterisk represents the statistical differences.

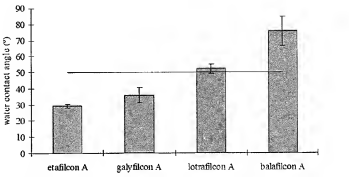

FIGURE 2.

Water contact angle (ϑ) formed on silicone–hydrogel lenses (galyfilcon A, lotrafilcon A, and balafilcon A) and conventional hydrogel (etafilcon A). Hydrophobic surfaces have water contact angles above 50°. Bars represent the standard deviations.

DISCUSSION

In this study, adhesion to contact lenses was performed using a static slide method. This methodology of assessing adhesion has been controversial because of the use of washing steps necessary to remove nonadherent and loosely adherent cells.[13] It has been demonstrated that the passage of an air–liquid interface on adhered bacteria can remove some of the adhered bacteria.[14] However, when in the presence of a more hydrophilic substratum or for higher interface passage speeds, this effect is attenuated.[15] Cerca et al.[16] studied the adhesion of 11 clinical strains of *S. epidermidis* to acrylic and glass surfaces using several different washing procedures and demonstrated that when using hydrophilic glass, no effect was observed on the passage of the air–liquid interface. In the case of hydrophobic surfaces, that effect was sometimes observed but attenuated by rapid washing to minimize the time of exposure of the adherent cells to the air–liquid interface.

The most common approach to enumerate adherent bacteria relies on the removal of the organisms from the lens surface followed by viable cells culturing. The method used in this study is based on the direct enumeration of adhered cells.[17] This technique is advantageous over other methods that use vortexing or sonication to remove adhered cells in ensuring that all adhered bacteria are quantified. Additionally, on account of microbial aggregation (very common on *Staphylococci* species), colony-forming units usually underestimate the number of cultivable bacteria.

The incorporation of silicone into a hydrogel polymer gives the advantage of a high oxygen transmissibility, but the disadvantage of decreased hydrophilicity.[18] To render the surface hydrophilic, techniques incorporating plasma into the surface of the lens have been developed. In the case of lotrafilcon lenses, they are permanently modified in a gas plasma-reactive chamber to create a continuous hydrophilic surface.[1,19] However, the water contact angles measured on the surface of this lens revealed a hydrophobic surface (Fig. 2). In the case of balafilcon, the lens surface is treated in a gas

plasma-reactive chamber, which transforms the silicone components on the lenses surface into hydrophilic silicate compounds, resulting in the formation of "silicate islands."[1,19] Between this, silicate islands are hydrophobic areas, which may explain the high water contact angle formed on the surface of this lens (Fig. 2) and thus its higher hydrophobicity compared with lotrafilcon. Galyfilcon A has no surface modification, but incorporates a moisture-rich internal wetting agent brandnamed HYDRACLEAR, based on PVP (polyvinyl pyrrolidone), that provides a hydrophilic layer at the surface of the material, which reduces the degree of hydrophobicity.[2]

The two extended wear silicone–hydrogel lenses have a surface hydrophobicity higher than that of conventional hydrogel lens and the daily wear silicone–hydrogel lens. These differences in surface hydrophobicity may explain the differences found in bacterial adhesion. Many studies have suggested that hydrophobic surfaces are more prone to pathogens adhesion than hydrophilic ones.[15,20] Beattie et al.[21] studied *Acanthamoeba* attachment to a silicone–hydrogel lens (balafilcon A) and conventional hydrogel contact lenses and concluded that balafilcon A is more prone to bacterial adhesion. These authors suggested that the high levels of attachment found in silicone–hydrogel lenses may be the result of the inherent property of the polymer or of the surface treatment procedure that originates in areas of hydrophobic material unoxidized after treatment.

In the present study, it was found a significant higher extent of adhesion of *P. aeruginosa* to the silicone–hydrogel lenses than to the conventional hydrogel lens (Fig. 1), with the exception of galyfilcon A. Willcox et al.[22] also found an increased capability of *P. aeruginosa* to adhere to silicone–hydrogel balafilcon A when compared with the adhesion to conventional hydrogels. Conversely, Borazjani et al.[23] found no significant differences between the adhesion of *P. aeruginosa* to silicon–hydrogel balafilcon A and etafilcon A. These contradictory results may be the result of the different bacterial strains used and growth conditions used. Several authors have reported that the extent of *P. aeruginosa* adherence is strain-dependent and influenced by growth stage and media.[22,24-26]

The ability of *S. epidermidis* 9142 to adhere to the hydrophobic silicone–hydrogel lotrafilcon A and balafilcon A was also greater than to the hydrophilic etafilcon A and hydrophilic silicone–hydrogel galyfilcon A, reinforcing the idea that hydrophobic silicone lens are more prone to bacterial adhesion.

The same conclusions could not be drawn for the strain 12,228, which is considered a biofilm-negative strain. Despite of being hydrophilic, galyfilcon A was equally prone to *S. epidermidis* 12,228 adhesion as the other hydrophobic silicone–hydrogel lenses. However, because this strain exhibited very low adherence capabilities, the differences observed in the extents of adhesion were not statistically relevant. Garcia-Saenz et al.[27] also reported low adhesion extents of a biofilm positive strain of *S. epidermidis* to contact lenses. The low adherence ability of this strain, compared with the biofilm-positive 9142, may be related to the absence of the *ica* operon in strain 12,228.[11] It is well documented that genes contained in the *ica* locus are responsible for the production of poly-N-acetyl-glucosamine (PNAG) and that PNAG is important for biofilm formation and adhesion to catheters.[28,29]

It must be stressed that the adhesion studies were carried out on the native polymer of contact lenses. However, it should be considered that *in situ* contact lenses become rapidly conditioned with the tear film proteins and mucins, which may modulate bacterial adhesion either by altering lens surface properties like hydrophobicity or by inducing the establishment of specific interactions between tear molecules and microbial cell receptors.[25] Nevertheless, this study may provide an indication of the likely transference of bacterial cell from the wearer's fingers to the contact lenses surfaces. On the other hand, Borazjani et al.[23] found no marked differences in the adhesion of *P. aeruginosa* to worn and unworn silicon–hydrogel lenses, suggesting that these lens surface properties were not affected by 6 to 7 days extended wear and thus by the presence of tear film molecules.

Summarizing, this *in vitro* adhesion studies revealed that silicone–hydrogel are more prone to bacterial colonization than conventional hydrogel lens. The exception was found for galyfilcon A that exhibited the same degree of adhesion of etafilcon A as a result of its low hydrophobicity. On the basis of this data, it could be speculated that the risks associated with silicone–hydrogel extended wear, when regarding microbial adhesion, would be higher than conventional hydrogel and daily wear silicone–hydrogel. However, the increased oxygen transmissibility of silicone–hydrogel lenses reduces corneal hypoxia and diminishes tissue damage, leading to reduced bacterial binding to corneal epithelium cells.[30] Thus, the drawbacks of lens colonization are minimized and the safety of extended wear of this type of lens is improved.

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Annex 6

About the ATCC-LGC Standards Partnership
LGC Standards Offices
How to Order
Special Forms
Technical Support
ATCC Cultures and Products
ATCC Science
ATCC Standards
ATCC Deposit Services
ATCC Custom Services
ATCC Product Use Policy

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

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Bacteria

ATCC® Number: **8014™** Order this Item **Price:** **£228.00; €298.00**

Organism: *Lactobacillus plantarum* (Orla-Jensen) Bergey et al. deposited as *Lactobacillus arabinosus* Fred et al.

Designations: 17-5 [BUCSAV 217, BUCSAV 449, Glaxo 664, ICPB 2080, NCDO 82, NCIB 6376, NCIB 8014, NCIB 8030]

Depositor: E McCoy

Biosafety Level: 1 **Shipped:** freeze-dried

Growth Conditions: ATCC medium 416: Lactobacilli MRS broth
Temperature: 37.0°C

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Products

Cross References: Nucleotide (GenBank) : AF189765 Lactobacillus plantarum alpha-galactosidase (melA) gene, complete cds.
Nucleotide (GenBank) : U97139 Lactobacillus plantarum 16S/23S ribosomal RNA large intergenic spacer region, tRNA-Ile and tRNA-Ala genes, complete sequence.
Nucleotide (GenBank) : X62346 L.plantarum plasmid 8014-2 DNA (from BclI to HincII site).

4. Examine the plates. If no growth has occurred then incubation should be continued up to 5 days before plates are discarded, as up to 20% of non-sporing anaerobes require prolonged incubation under unbroken anaerobic conditions.
5. Carry out confirmatory tests on the isolates and record the results as follows:
   (a) All facultative anaerobes and anaerobes isolated on the Wilkins-Chalgren Anaerobe Agar Medium plate (Plate 1).
   (b) All non-sporing anaerobes isolated on the medium for non-sporing anaerobes (Plate 2).
   (c) All Gram-negative anaerobes isolated on the medium for Gram-negative anaerobes (Plate 3).

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Nutrient Agar

Code — Powder CM3
Tablets CM4

FORMULA

	grams per litre
'Lab-Lemco' Powder	**1**
Yeast Extract	**2**
Peptone	**5**
Sodium chloride	**5**
Agar	**15**

pH 7.4 ± 0.2

DIRECTIONS

Powder: Suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.
Tablets: Add 1 tablet to 5 ml of distilled water and soak for 5 minutes. Sterilize by autoclaving at 121°C for 15 minutes.

DESCRIPTION

A simple medium, one of the first in the Oxoid range, and one which has repeatedly proved its worth; amongst the many published works which mention the Oxoid medium there are accounts of the cultivation of members of the following genera: *Alysiella, Agrobacterium, Arthrobacter, Bacillus, Brucella, Escherichia, Flavobacterium, Micromonospora, Pseudomonas, Rhizobium, Sarcina, Simonsiella, Staphylococcus, Streptomyces, Thermopolysora, Thiobacillus* and *Xanthomonas.*

Nutrient Agar is an inexpensive medium and so is suitable for teaching and demonstration purposes. It contains a concentration of 1.5% of agar to permit the addition of up to 10% of blood or other biological fluid, as required. The medium, without additions, may be used for the cultivation of organisms which are not exacting in their food requirements.
For a medium which is richer in nutrients, see Blood Agar Base No. 2 CM271.

Nutrient Broth

Code — Powder CM1
Tablets CM2

FORMULA

	grams per litre
'Lab-Lemco' Powder	**1**
Yeast Extract	**2**
Peptone	**5**
Sodium chloride	**5**

pH 7.4 ± 0.2

DIRECTIONS

Powder: Add 13 g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.
Tablets: Add 1 tablet to 10 ml of distilled water, and sterilize by autoclaving at 121°C for 15 minutes.

DESCRIPTION

A very economical medium for the cultivation of organisms which are not exacting in their food requirements. 500 grams of Oxoid Nutrient Broth powder makes over 38 litres of broth. The reconstituted and sterilized medium may be enriched with other ingredients such as carbohydrates, blood, serum, etc. as required for special purposes. See also Nutrient Broth No. 2 CM67.

Nutrient Broth No. 2

Code — Powder CM67
Tablets CM68

FORMULA

	grams per litre
'Lab-Lemco' Powder	**10**
Peptone	**10**
Sodium chloride	**5**

pH 7.5 ± 0.2

DIRECTIONS

Powder: Add 25 g to 1 litre of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121°C for 15 minutes.
Tablets: Add 1 tablet to 10 ml of distilled water and sterilize by autoclaving at 121°C for 15 minutes.

Annex 7